METHOD FOR DISCOVERING NEUROGENIC AGENTS

The present patent application claims the benefit of U.S. Provisional Patent Application 60/432,359, filed December 9, 2002, and U.S. Provisional Patent Application 60/493,674, filed August 8, 2003, which are hereby incorporated by reference in their entirety and relied upon.

BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

According to a long-held doctrine, no significant number of neurons are made and contribute to function in the adult mammalian nervous system. However, recent data indicate that adult mammalian brains contain neural precursor cells capable of generating new neurons both in normal and in injured conditions. These new neurons have been quantified in live animals by injecting or feeding in drinking water a marker of dividing cells, bromodeoxyuridine (BrdU) and by immunostaining of post-mortem brains with antibodies against BrdU and neuronal markers. An endogenous marker of dividing cells, ki67 protein, has also been used instead of BrdU for this

purpose. Thus, in healthy, young rodents, approximately 3,000 - 15,000 new cells per day are estimated to be born in the dentate gyrus of the hippocampus, about 60% of which express early neuron-specific proteins such as double-cortin and type III beta-tubulin. Significant number of new cells and new neurons have also been observed in healthy, young primates. In rodents as well as in primates, the location of neurogenic areas in the CNS is limited to the dentate gyrus of the hippocampus and the subependymal layer of the striatum. In human patients of different ages who have been diagnosed with a tumor of the tongue, a single injection of BrdU has revealed significant number of new cells and new neurons being born in the dentate gyrus and the subependymal layer of the striatum. Thus, the process of generating new neurons (neurogenesis) occurs in the mature, adult brain in significant quantities throughout rodents, primates, and human species.

Such significant quantities of new neurons suggest that they may be important for the normal physiology of the brain, especially the hippocampus. Hippocampus is the well-known center of learning, memory, and other cognitive functions, processes which new information are added, edited, stored, and recalled constantly throughout life.

Since hippocampus is also the most potent neurogenic area of the brain, many studies have been undertaken to establish whether neurogenesis may be the cellular mechanism to structurally accommodate the ever-increasing volume of cognitive processing to be handled. Thus, it has been shown that at least some of the newly born neurons, marked by genetic markers, do mature to be electrophysiologically active and integrate into the existing neuronal circuitry of the hippocampus. Ablation of the neurogenesis in rats leads to decreased cognitive capabilities in several behavior tests. Thus, the existing data demonstrate that neurogenesis significantly contributes to the normal hippocampal physiology.

In abnormal conditions, such as when an injury to a brain area has occurred, neurogenesis becomes more wide-spread and perhaps functionally diverse. In rodent models of ischemic and hemorrhagic stroke, the newly born neurons of the subependyma (also referred to as subventricular zone) are seen migrating to and accumulating in the lesion area of the cortex. However, the newly born neurons have short survival period.

Thus, a compound that can stimulate the endogenous neurogenesis either in a disease state or in a healthy

state may be an effective drug for a number of human nervous system diseases. However, the current limitation is the lack of an effective, predictive in vitro assay that can be used to select a neurogenic compound for clinical drug development. Disclosed here is a novel, in vitro assay, which is effective and predictive, to be useful for discovering a compound that promotes neurogenesis in vivo. Also disclosed are classes of compound structures that are shown to be particularly effective in promoting the neurogenesis.

This invention relates to the method of discovering a neurogenic drug to treat neurologic, psychiatric, and aging-related disorders. It also relates to the use of Fused Imidazoles, Aminopyrimidines, Nicotinamides, Aminomethyl Phenoxypiperidines and Aryloxypiperidines for use as therapeutic agents and analytical reagents by means of promoting neurogenesis. More particularly this invention relates to these agents as therapeutics for prevention and treatment of neurological diseases in mammals and reagents for detecting neurogenesis and proliferation.

2. DESCRIPTION OF THE RELATED ART

Most antidepressants are thought to work by increasing the levels of monoamines available for post-synaptic receptors. Examples of classes of agents working apparently by the "monoaminergic hypothesis of depression" include the selective serotonin uptake inhibitors (SSRIs) like fluoxetine, the mixed noradrenaline/serotonin transporter blockers like tricyclic agent imipramine and noradrenaline uptake inhibitors like desipramine. The antidepressant-induced increase in intraneuronal biogenic amines occurs quite rapidly. However, the antidepressant-induced improvement in clinical behavior requires weeks of daily administration.

One hypothesis that may account for the slow-onset of the antidepressants' therapeutic activity is that they work by promoting hippocampal neurogenesis. It is expected that neurogenesis would require a number of weeks for stem cells to divide, differentiate, migrate and establish connections with post-synaptic neurons. The neurogenesis theory of depression then postulates that antidepressant effect is brought about by structural changes in the hippocampal circuitry contributed by newly generated neurons stimulated

by antidepressants (Malberg et al., 2000; Czeh et al, 2001; Santarelli et al, 2003).

The neurogenic theory of depression, though not conclusive, has strong supportive data including the finding that neurogenesis is actually requisite for antidepressant behavioral improvement in the novelty suppressed feeding model (Santarelli et al., 2003). A therapeutic benefit from hippocampal neurogenesis is further supported by the finding of hippocampal atrophy in depression, where MRI imaging studies identified a reduction in the right and the left hippocampal volumes in individuals with major depression (Sheline et al., 1996; Bremner et al., 2000; Mervaala et al., 2000). Long standing works also suggest a strong relationship between glucocorticoid dysregulation or glucocorticoid hypersecretion in stress and depression, such that the hippocampal volume loss might be considered a consequence of glucocorticoid-induced hippocampal neuronal loss (Sheline et al., 1996; Lucassen et al., 2001; Lee et al., 2002 (review)). Furthermore, in studies which involved the administration of a chronic stress to animals, both hippocampal volume changes and reduction in neurogenesis were observed, and these events were both reversed by

chronic antidepressant administration (Czeh et al., 2001; Pham et al., 2003), further illustrating the strong association between depression, stress and neurogenesis. The association comes full circle, since agents or conditions that promote a reduction in neurogenesis also appear as causative agents/events in depression, specifically glucocorticoid (Sapolsky, 2000), and depletion of serotonin (Brezun and Daszuta, 1999). Kempermann and Kronenberg (2003), though acknowledging the validity of the hippocampal neurogenesis theory of depression, suggest that this hypothesis needs to be looked at in the context of a larger model of cellular plasticity, which elucidates how antidepressants induce nascent neurons of unknown phenotype to survive and produce viable circuits.

Neurogenesis can be characterized as three successive stages: proliferation of endogenous stem cells and precursors, differentiation into neurons and neuron maturation with formation of viable synaptic connections (plasticity). By taking into account all stages of neurogenesis, then the hippocampal volume loss in depression could potentially be caused by 1) inhibition of the endogenous hippocampal stem cell proliferation in the dentate gyrus, 2) inhibition of differentiation and

dendrite development and 3) by loss of neurons (apoptosis) and their dendritic structure. Though apoptosis is observed in depression, hippocampal apoptosis as measured by DNA fragmentation from depressed patients appears to play only a minor role in the volume loss (Lucassen et al., 2001). In an animal model of acute stress or in normal animals receiving exogenous corticosterone the stress did cause a reduction in synaptic plasticity in the hippocampus (Xu et al., 1998). Chronic administration of the tricyclic antidepressant, imipramine partially reversed the loss in LTP in socially stressed, depressive-like animals (Von Frijtag et al., 2001) suggesting imipramine effects on the plasticity phase of neurogenesis. In another animal model of depression, presenting neurogenesis loss and hippocampal volume loss, stressed animals that chronically received the antidepressant, tianeptine, showed similar numbers of dividing cells as control animals (no social stress) a measure of proliferation (Czeh et al., 2001). In an experiment looking at association of antidepressants and neurogenesis in normal adult rats, the antidepressant, fluoxetine required chronic administration to cause proliferation of cells in dentate gyrus (24 hrs post treatment), but there was considerable loss of nascent

cells whether in the presence or absence of fluoxetine treatment, where fluoxetine provided no observed differentiation or survival benefit (Malberg et al.,2000). Results on different neurogenic intervention points by known antidepressants suggest that novel neurogenic agents that intervene at different points in the neurogenesis pathway, could result in potentially diverse therapeutic effects on depression.

These points of intervention can be studied and the target elucidated for novel antidepressant candidates through in vitro assays. Since adult stem cell proliferation and neurogenesis is observed in adult vertebrates in hippocampal dentate gyrus (Gould et al., 2001; Eriksson et al., 1998), we can use multi-potential hippocampal stem cells to screen agents in vitro for neurogenic activity.

Interestingly, chronic administration of either the antidepressant fluoxetine, an SSRI or the antidepressant rolipram, a phosphodiesterase IV inhibitor, promoted neurogenesis in normal animals (Malberg et al., 2000; Nakagawa et al., 2002). One might conclude from these results that any agent that promotes neurogenesis will generate a behavioral benefit in depression, unrelated to

the agents mechanism-of-action or possibly there is a common pathway where both drug actions overlap. D'Sa and Duman (2002) suggest a scheme whereby the phosphorylation and activation of CREB and the subsequent expression of BDNF are central to the induction of neurogenesis, that culminates in antidepressant behavior. CREB phosphorylation is increased in animals administered rolipram chronically (Nakagawa et al., 2002) and antidepressants that either increase Ca2+/CaM-kinases or cAMP could cause the phosphorylation of CREB in the nucleus (reviewed by D'sa and Duman 2002). They further suggest that the phosphorylated CREB then binds to CRE binding site to promote the expression of BDNF and bcl-2, that appear critical to cell survival and plasticity. Proof of neurotrophic factor BDNF's involvement in depression comes from studies showing that antidepressants and electroconvulsive shock both caused an increase in BDNF levels (Nibuya et al., 1996) and that intrahippocampal injection of BDNF had antidepressant activity in two models of depression (Shirayama et al., 2002).

If neurogenesis is critical for antidepressant activity is it also sufficient and is the mechanism by which the neurogenesis occurs or timing of neurogenesis also critical

to the therapeutic activity? We can try to answer these questions using novel agents developed through screening paradigms that identify agents that promote the proliferation and differentiation of endogenous hippocampal stem cells to neurons in vivo if they will be effective antidepressants. Since we have completed the screening of 10,000 small molecule compounds in in vitro models of neurogenesis and shown that our in vitro screen is predictive of in vivo neurogenic efficacy, we can then test these orally available agents, that promote in vivo neurogenesis, in models of depression. Rolipram, an antidepressant that works by increasing cAMP levels and is neurogenic in animals (Nakagawa et al., 2002) was effective in our primary in vitro neurogenesis screen. This suggests that our primary in vitro screen would include those agents that might promote neurogenesis by targeting the cAMP/pCREB/BDNF pathway. This does not necessarily exclude all other neurogenesis mechanisms for our NSI compounds. If the target of these neurogenic agents are important for behavioral activity where three separate chemically diverse classes showed in vitro assay efficacy differences and that the mechanism for all does not overlap at the point of CREB phosphorylation and BDNF expression then we might expect

very different effects on behavioral activities in depression models.

Neuropathology associated with key cognitive regions of the Alzheimer's diseased brain have led to therapeutic strategies that address the neuronal loss, in the hopes of reducing the cognitive decline. One strategy enlists trophic agents, that regulate neuronal function and survival, as AD therapeutics (see Peterson and Gage, 1999). Problems with systemic administration, side effects and locating trophic-sensitive neurons generated few clinical successes with these therapies. One AD therapeutic, AIT-082, promotes memory enhancement in AD individuals potentially by stimulating endogenous trophic factors (Ritzman and Glasky, 1999; Rathbone et al., 1999). So the use of agents to promote increased survival and function of the remaining available neurons appears to have some therapeutic value.

Hippocampus is one of the main brain regions where neurogenesis in adult brain has been documented across several vertebrate species, including monkeys and humans (e.g., Gould et al., 2001; Eriksson et al., 1998). In fact, adult hippocampal neurogenesis contributes functionally to cognitive capacity. Shors et al. (2001) reported that inhibition of

neurogenesis in adult rat hippocampus, in the absence of the destruction of existing neurons, caused impaired memory function. Many studies observed that degenerative conditions induced neurogenesis in mature mammalian brains, suggesting the existence of a natural repair pathway by means of neurogenesis. A focal ischemic model, reversible photothrombic ring stroke, caused increased neurogenesis in rat cortex by 3-6% (Gu et al., 2000). Seizures induced by electroconvulsive shock in adult rats increased neurogenesis in dentate gyrus of hippocampus (Scott et al, 2000; Madsen et al, 2000). Also, rats gamma-irradiated to kill mitotic cells in the CNS showed reduced numbers of nascent neurons and reduced LTP in slice cultures. These observations highlight the likelihood that a cellular mechanism for neurogenesis within adult human CNS, especially in hippocampus, does exist both as a normal physiological process and as a self-repairing pathway.

In adult neurogenesis a decline due to aging is observed (Kuhn et al., 1996), though proof that this age-dependent decline in neurogenesis causes cognitive impairment is still debated. Considerable evidence does exist, indicating that increased neurogenesis reduces age-associated cognitive decline. This is most dramatically observed with the

transplantation of human stem cells into aged rats initiating improved water maze learning and retention (Qu et al., 2001). Other data suggests that induction of neurogenesis by diet restriction (Lee et al., 2000) exercise (van Praag et al., 1999) or growth factor addition (Lichtenwalner et al., 2001) improves learning and memory in adult or aged rats. A number of other inducers of neurogenesis have been identified, including anti-depressants (Malberg et al., 2000; Czeh et al., 2001), and nitric oxide donors (Zhang et al., 2001) suggesting the usefulness of neurogenic agents for other diseases presenting cognitive-deficits, such as stroke and depression. A small molecule that induces hippocampal neurogenesis that is blood brain barrier penetrable would allow for a potentially novel oral therapeutic for Alzheimer's disease.

Other potential AD therapeutics progressing in clinical trials, target neurodegeneration in the hopes of reducing the neuronal loss and cognitive decline. Apoptotic death involving caspase pathways and DNA fragmentation has been measured in *in vitro* and animal models of AD and in Alzheimer's diseased brain tissue. The extent of apoptosis leading to neuronal loss is of continual debate with most agreeing it has some effect, but that other neuronal death pathways definitely play a role

(see Behl, 2000; Broe et al., 2001; Roth, 2001). Concern that measures of upstream caspase markers in neurons from AD tissue may not proceed to degeneration has been suggested (Raina et al, 2001). In order to screen for a neuroprotectant therapeutics it is critical, therefore, to measure more than one endpoint of neuronal death and determine at what point an agent may intervene in the death pathway(s). Behl (2000) suggested that AD pathology is most likely a mixture of apoptotic and necrotic pathways and that concentrating therapeutic discovery using only one pathway may provide inconclusive results. All hits in our neurogenesis models were tested through our secondary apoptosis/necrosis assay to screen for agents that function both as neurogenic and neuroprotective agents. These agents may improve or reverse the cognitive decline observed in MCI and AD.

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SUMMARY OF THE INVENTION

A neurogenic drug is an agent that enhances the process of generating new neurons (neurogenesis). Recent studies indicate that neurogenesis occurs in the adult human brains under normal as well as under degenerative conditions and that such adult-generated neurons do contribute functionally to the brain physiology such as learning and memory. These observations highlight the likelihood that a cellular mechanism for neurogenesis within adult human CNS, especially in hippocampus, does exist both as a normal physiological pathway and as a self-

repairing pathway. What is lacking and contributes to permanent damage may be (1) the volume/persistence of neurogenesis and/or (2) the survival/maturation of the new neurons. The objective of the neurogenesis screen as described here is to discover a compound that will significantly boost either of these processes.

Many neurological diseases, including Alzheimer's disease, mild cognitive impairment, dementia, age-related cognitive decline, stroke, traumatic brain injury, spinal cord injury and the like are neurodegenerative conditions. Neuropsychiatric diseases including depression, anxiety, schizophrenia and the like also show nerve cell dysfunction leading to cognitive, behavioral, and mood disorders. A neurogenic drug would be beneficial for countering and treating these diseases.

The present invention discloses a method of discovering such a neurogenic drug. Such drug will serve to prevent or treat neurodegenerative and neuropsychiatric disorders by promoting the birth of new neuron endogenously within the nervous system by administering the compounds of the present invention into the patient. This may involve delivery of the agents alone or together with transplanted stem cells or progenitor cells.

Using the method herein, compounds of the type, Fused Imidazoles, Aminopyrimidines, Nicotinamides, Aminomethyl Phenoxypiperidines and Aryloxypiperidines are evaluated for their ability to promote neurogenesis by proliferation/ differentiation of human hippocampal multipotent stem/ progenitor cells and neuronal progenitors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic description of neurogenesis processes captured in the assay and different potential sites of a neurogenic drug action.

Figure 2. Detection of changes in cell number by Alamar Blue dye. Alamar Blue, a fluorescent dye, is used as an indicator of metabolic respiration to determine optimum plating density. Results at an initial plating density of 30,000 cells/well suggest a large difference in cell number on removal of mitogen from the N2b media (differentiation) versus N2b with mitogen (proliferation) conditions. This figure only describes total cellular activity, further markers are required to determine what cell types (e.g. neuronal, glial) are observed under differentiating media.

Figure 3A. Influence of known growth factors on proliferation and neurogenesis relative to control. Hippocampal progenitor cells were treated for seven days with differentiation media (without mitogen) in the presence or absence of 20 ng/ml of growth factor dosed every other day. Plates were treated with Alamar Blue as described in Methods, then fixed and stained with antibody (TuJ1) against type III beta-tubulin (neuronal marker). The 96-well plate was read in a fluorescent plate reader. Bars represent the Mean+SD from 4 wells per treatment.

Figure 3B. LIF effects on hippocampal cell proliferation and neurogenesis by manual cell counting. Hippocampal progenitor cells were treated for seven days with differentiation media (without mitogen) in the presence or absence of 20 ng/ml LIF. Three fields were analyzed per well for total number of cells (DAPI positive nuclei) and for total number of neurons (TUJI positive cells). Bars represent the Mean+SD from 4 wells per treatment. The percentage of neurons calculated for each treatment are as follows: 48.5 + 6.3% for controls and 53.6+1.15 for LIF. The non-TUJ1 positive cells are mainly astrocytic (GFAP+).

Figure 4. Examples of proliferation profile of compounds selected from primary screening. Proliferation was measured after compound treatment for 7 days by Alamar Blue staining of live cells per well. Shown are relative values over the vehicle control.

Figure 5. Example of neurogenesis profile of compounds selected from primary screening. After 7 days of compound treatment, the ratio of neuron number (TuJ1 stained) to the total nuclei number (Hoechst stained) was determined. Shown are the relative ratio of neuron:total cells for each compound over the vehicle control in percentage. Typical ratio for vehicle control is 40-50% neurons. The ratio can change by either increased differentiation of the cells to neurons, decreased proliferation of astrocytes, or increased proliferation of neuronal progenitors.

Figure 6. Examples of neurogenesis profile of compounds selected from primary screening. After 7 days of compound treatment, the cells were stained with TuJ1 for neurons. The absolute number of TuJ1+ neurons per area was quantified and expressed as a relative value to the vehicle treated control.

Figure 7. Dose-dependent increase in neuron number. Differentiating human hippocampal progenitor cells were treated for 7 days with varying concentrations of "primary hits". Subsequently, the cells were fixed, stained with TuJ1, and positive cells were quantified by an automated cell counter. Shown are the number of neurons after each treatment normalized against the vehicle control (0 microM =1.0).

DETAILED DESCRIPTION OF THE INVENTION

1. A Stable Cell Line of Neural Progenitors

A screening of a large number of unknown agents (e.g., protein factors, peptides, nucleic acids, natural compounds, or synthetic compounds) for discovering a candidate drug involves repeating the same test for several hundreds to several million times. This requires a great deal of reproducibility from the test. In order to obtain such reproducibility for neurogenesis assay, we have created stable cell lines of neural progenitors, which upon differentiation generate reproducible quantities of neurons. In a preferred embodiment, a multipotent neural stem/progenitor cell line derived from human hippocampus

was used. Cell lines derived from other CNS areas, including dentate gyrus of an adult brain, can also substitute. A neural progenitor population derived as a stable cell line from partial differentiation of embryonic stem cells can also be used. For this purpose, a cell line is defined as a population of cells having been expanded for at least 10 cell-doublings.

Cell lines that are genetically engineered to enhance the cells' mitotic capacity can also be used. In a preferred embodiment, the genetic modification consists of over-expression of functional c-myc protein intracellularly under a conditional activation system such as c-myc protein fused to a ligand-binding domain of an estrogen receptor. Cell lines that are not genetically engineered are preferred and can also be used.

In a preferred embodiment, a progenitor population that upon differentiation generates both neurons and glia in a single culture has been used. Presence of glia, either astrocytes and/or oligodendrocytes or their precursors, are required to promote physiological maturation of nascent neurons born from their precursors in culture.

In a preferred embodiment, differentiation of the progenitors is initiated by withdrawing the mitogen from the culture. Serum as well as other growth-promoting factors should be avoided from the differentiating culture since they will significantly affect the reproducibility and interfere with the neurogenesis assay.

2. Preparation of Assay Plate

Neural stem/progenitor cells differentiate spontaneously in the absence of a mitogen. Undifferentiated mitotic cells are harvested by enzyme treatment to remove residual mitogen, in the preferred embodiment, basic fibroblast growth factor (bFGF). The collected cells are seeded into appropriate plates (standard 96-well or 384-well) pre-coated with the usual extra cellular matrix proteins (poly-D-lysine and fibronectin, for example) for attachment of the cells. The initial seeding density can be within the range of about 2,000-125,000 cells per well of a 96-well plate. The preferred density is 40,000 cells per well of a 96-well plate, which has been optimized for best signal-to-noise ratio. Too low cell density retards the initiation of differentiation and results in poor plating efficiency,

which interferes with the assay. Too high cell density leads to inhibition of neurogenesis due to cell-cell contact and paracrine factors, which also interferes with the assay. The actual cell number can be proportionally decreased or increased depending upon the surface area of the culture substrate used. For example, for a 384-well plate, which has approximately 1/4 of the surface area of a 96-well plate, the initial seeding density should be decreased accordingly (1/4).

3. Detection of Neurogenesis

The key activity of a neurogenic drug is to increase the number of neurons generated from their precursors. A molecule can bring about such increase in the neurogenesis by a number of different mechanisms. It can act as a mitogen for the neural stem/progenitor cells and increase the progenitor's cell number, which in turn results in increased number of neurons in the culture when differentiated. Or, it can act as a neuronal specification factor by promoting the stem/progenitor cell differentiation toward neurons in the expense of glia. This will also result in increased number of neurons in the culture, but without changing the overall cell number. Or,

it can act as a mitogen for committed neuronal progenitors that differentiate only into neurons. Increasing this subpopulation would also increase the final number of neurons in the culture. Or, it can act as a survival factor to rescue immature neurons from undergoing cell death during differentiation, which will result in increased neurons (Figure 1).

The assay method here captures all of these possibilities by allowing for sufficient time for these processes to unfold. In a preferred embodiment, for human neural stem/progenitor cells, the assay is continued for seven days. A minimum of three days from the onset of differentiation should be allowed for stable expression of definitive neuronal markers to appear. A sufficient time is also required for a compound action on differentiation and/or proliferation to take place to a sufficient degree to be reliably detectable. Manifestation of drug-induced changes in neuron number takes a minimum of three days for the human cells to be detectable.

The final neuron number is detected by immunostaining of the culture with antibodies against neurons and quantified by counting of the immunopositive neurons and/or by measuring the staining intensity.

4. Method for Measuring Neurogenesis

(1) Undifferentiated human neural stem/progenitor cells were harvested by enzyme treatment.

(2) The collected cells were seeded at 40,000 cells per well of 96-well plates pre-coated with extracellular matrix proteins (e.g., Biocoat PDL, Fisher). The seeding media is a standard serum-free, growth factor-free, basal media that supports healthy neuronal/glial survival, such as N2 without phenol red.

(3) Test agents at appropriate concentrations were added to each well on Day 0.

(4) The assay plates were incubated for 7 days, with 50% media change at every other day. On Day 2, 4, and 6 of post-plating, additional increment of the screening agents at appropriate concentrations were added to each well.

(5) On the final day of the culture (Day 7), alamar blue dye was added to each well and the cultures were further incubated for 2 hours at 37^{0}C.

(6) The fluorescence of the oxidized dye in each well was read by a fluorescent plate reader with the following settings:

Read Mode End Point

Excitation 530nm, emission 590nm, cutoff 570nm

The fluorescence level is proportional to the number of respiring cells in the culture and is a measure of a proliferative activity of a test agent (Figure 2).

(7) After the alamar blue assay, the cells were fixed and stained with antibodies against neuron-specific antigens according to standard procedures. Typical antigens effective were TypeIII-beta tubulin and MAP2c.

(8) The total cell number in each well was quantified by staining the cultures with a nuclear dye such as DAPI or Hoechst according to standard procedures.

(9) As a preliminary detection of positive activities, the overall immunostaining intensity in each well was read by a fluorescence plate reader. For the positive hits, more quantitative analysis was carried out by automated morphometric counting of individual cells.

5. Examples

Example 1. Selection of a positive control. Several neurotrophic factors--including brain-derived neurotrophic factor, glia-derived neurotrophic

factor-3, and leukemia inhibitory factor--suggested to have neurogenic properties were tested in the assay described above. Only one (leukemia inhibitory factor) was effective (Figure 3A and 3B). Thus, the assay can discriminate test agents for selectively having a neurogenic activity. The positive control utilized is leukemia inhibitory factor (LIF), a cytokine growth factor, at 20 ng/ml. The selection of LIF as the positive control is based on its properties to increase by 2-3 fold the number of neurons and glia. This effect validates both the neural stem cell system, in which, should a compound be effective in neurogenesis, the cells respond appropriately by enhanced differentiation and/or mitosis, and the assay method in which such cellular responses can be measured reproducibly and quantifiably.

Example 2. Primary screening of unknown compounds.

5,628 synthetic compounds of the type Fused Imidazoles, Aminopyrimidines, Nicotinamides, Aminomethyl Phenoxypiperidines and Aryloxypiperidines are evaluated for their effect on neurogenesis according the assay method described above. From the preliminary analysis using the fluorescent plate reader, over 300 compounds to date showed initial positive activity. Those were re-analyzed by quantitative neuron counting. Among them, 30 compounds

significantly increased cell number ("proliferation", Figure 4); 53 increased the number of neurons ("neurogenesis", Figure 5 & Figure 6); and 7 showed significant activity in both. The significance level was empirically set at an activity above 30% change over the vehicle control for proliferation and above 10% change for neurogenesis. A summary of the result in the compound screening is provided in Table I.

Table I. Summary of Compound Screening

Primary Screen	Hits Confirmed	Proliferation Hit	Neurogenesis Hit	Double Hit
0	0	0	0	0
2,240	88	13	8	1
5,628	>300	30	53	7

Example 3. Dose-response profiles.

Linearity of dose-response and in vitro neurotoxicity are used to further filter down desired compounds from the primary screen. The dose-response curve measures neurogenesis over a concentration range of 100picaM to 100microM. The rationale for this is to eliminate early on those compounds with pronounced toxicity and those without a dose-dependent effect on neurogenesis. Examples of several primary hits fully analyzed for dose-response are

shown in Figure 7. Significantly, most compounds exhibit a linear response over several log concentrations below 1microM. This indicates that the assay for primary screening is reliable and that the quality of the compound library is high. Table II contains the summary of EC50 of each compound tested. On the other hand, at high concentrations (100 microM), some, but not all showed high level of neurotoxicity, indicating that analyzing dose-response curves will be discriminatory and serve as an effective early filter.

Table II. Activity Profile of Primary Hits in Vitro

Compound ID	Proliferation (% of Control)	Neuron Ratio (% of Control	EC50 for Neuron Number	Other Characterization Of Toxicity
NSI-106	211 ± 48	92 ± 6	0.1nM r^2 0.75	No Toxicity
NSI-144	149 ± 15	137 ± 8	1.0nM r^2 0.54	No Toxicity
NSI-152	174 ± 49	112 ± 4	0.1nM r^2 0.84	Toxic At Highest Dose
NSI-154	211 ± 63	102 ± 6	0.3nM r^2 0.79	No Toxicity
NSI-155	198 ± 44	118 ± 8	0.05nM r^2 0.49	Toxic At Highest Dose
NSI-163	208 ± 25	120 ± 11	1.0nM r^2 0.81	Toxic At Highest Dose

UTILITIES OF THE INVENTION

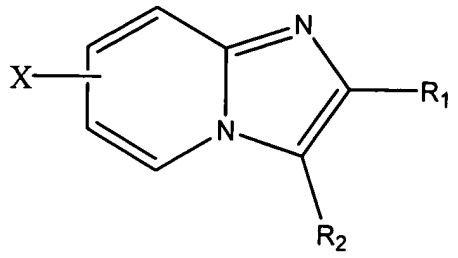
In one aspect of this invention an agent would be administered to treat a neurodegenerative disease. In a preferred embodiment of this invention the neurodegenerative disease would be Alzheimer's disease, dementia, mild cognitive impairment, aged-related cognitive decline, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, demyelination, stroke, spinal injuries, traumatic injuries, neuropathic pain, and the like.

In another of its aspects, this invention the agent would be administered to treat a psychiatric disease. In a preferred embodiment of this invention the psychiatric disease is depression, post-traumatic stress syndrome, stress, anxiety, schizophrenia, sleep deprivation, cogntive dysfunction, amnesia, and the like.

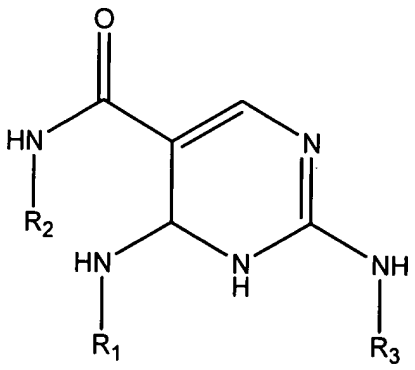
In another aspect of the invention an agent would be administered by any number of routes and multipotent stem cells or differentiated multipotent stem cells would be transplanted into brain.

In another aspect of the invention the structures of the formula are utilized in above methods:

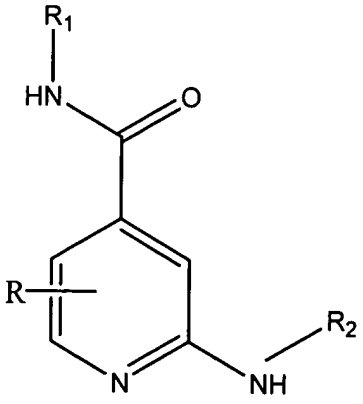
Structure Formula 1: fused imidazoles



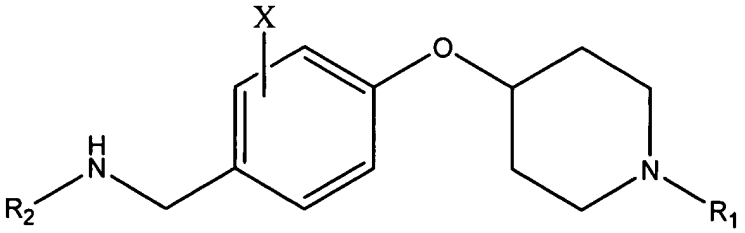
Structure Formula 2: aminopyrimidines.



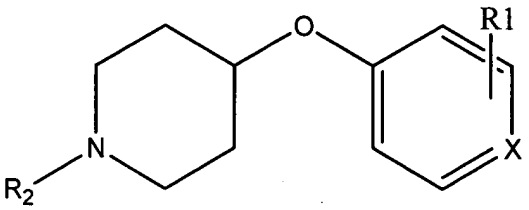
Structure Formula 3: nicotinamides



Structure Formula 4: aminomethyl phenoxypiperidines



Structure Formula 5: aryloxypiperidines



While the invention has been described in connection with what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention is not limited to the disclosed embodiments, but on the contrary is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus, it is to be understood that variations in the present invention can be made without departing from the novel aspects of this invention as defined in the claims.

All patents and articles cited herein are hereby incorporated by reference in their entirety and relied upon.